Microvascular Retinal Endothelial and Pericyte Cell Apoptosis In Vitro: Role of Hedgehog and Notch Signaling

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PURPOSE. Aberrant retinal blood flow is a hallmark of retinopathies and may be a causative factor in their pathophysiology. In this study, the effects of pulsatile flow on hedgehog and Notch control of retinal endothelial cell and pericyte apoptosis were examined.

METHODS. The levels of hedgehog and Notch signaling components in bovine retinal endothelial cells (BRECs) and pericytes (BRPs) were examined in vitro under static conditions and after exposure to pulsatile flow, with a perfused transcapillary co-culture system. Notch and hedgehog signaling was examined by immunocytochemistry, immunoblot, and real-time PCR.

RESULTS. Notch and hedgehog proteins were present in BRECs and BRPs in vitro and in human retinal vasculature in vivo. Inhibition of hedgehog with cyclopamine and Notch with DAPT decreased hedgehog target gene levels and Notch intracellular receptor expression, respectively, concomitant with an increase in BREC and BRP apoptosis. Sonic hedgehog (Shh) mediated upregulation of Notch1 receptor levels was attenuated after cyclopamine treatment in both cell types. Exposure of co-cultured BRECs and BRPs to pulsatile flow increased apoptosis in the BRPs while concurrently decreasing apoptosis in the BRECs. These changes were concomitant with increased expression of Notch and hedgehog signaling components in the BRECs and reduced expression in the BRPs. The flow-induced decrease in apoptosis in the BRECs was associated with increased Notch receptor expression and was reversed after inhibition of hedgehog signaling with cyclopamine and inhibition of Notch signaling after ectopic expression of the CBF-1/RBP-Jk-binding protein, RPMS-1.

CONCLUSIONS. Pulsatile flow promotes BREC survival and enhances BRP apoptosis through modulation of Notch and hedgehog pathways. These interactions have important implications for the pathogenesis of retinopathies. (Invest Ophthalmol Vis Sci. 2011;52:4472–4483) DOI:10.1167/iovs.10-7061

The retinal vasculature is composed of the inner and outer retinal vascular plexus and undergoes extensive reorganization during development or under specific pathologic conditions in adulthood.1 Vessel regression, sprouting angiogenesis, vascular remodeling, and cell differentiation events are involved and critically depend on paracrine and juxtacrine interaction and signaling between different cellular components, such as neurons, glia, endothelial cells, pericytes, and immune cells.1,2 Transgenic mouse studies have begun to unravel the genetic basis of some of these signaling pathways and have led to an improved understanding of the molecular mechanisms that control retinal blood vessel behavior. From these studies, it is clear that mice deficient in either Notch3,4 or hedgehog (Hh)5 components exhibit defective retinal microvascular development.

The Notch signaling pathway is a highly conserved developmental pathway that controls cell differentiation during embryonic development of the vasculature and is replicated in adult cells after vascular injury.6–9 Notch genes encode cell surface receptors that transduce signals between contiguous cells, to regulate developmental processes such as cell fate decisions.6–9 Mammals express four Notch receptors (Notch1 to –4) and 5 membrane-bound ligands (Jagged [Jag]1, Jag2, δ-like [Dll]1, Dll3, and Dll4). After ligand binding, Notch receptors undergo proteolytic cleavage by tumor necrosis factor-β-converting enzyme and the γ-secretase complex, to release the Notch intracellular domain (NICD). NICD then translocates to the nucleus, where it binds with the transcription factor CSL (CBF-1/RBP/Jk, Su[H], and Lag-1) and coactivator Mastermind-like (MAML) to trigger downstream target gene expression [Hrs]. In the vasculature, alterations in Notch signaling result in abnormalities in vessel patterning and maturation. Notch1 and 3 NICDs modulate macrovascular cell growth in response to growth factor stimulation and biomechanical activation.10–12 Notch signaling is significantly enhanced in low-cyclic-strain environments in vitro and in vivo,1,11,13,14 concomitant with increased macrovascular cell proliferation and survival.

Hh signaling is also known to have crucial roles in vascular development and angiogenesis.15 Hh’s are a class of 19-kDa morphogens that interact with heparin proteoglycans on the cell surface through an N-terminal basic domain. They are tethered to the cell surface through cholesterol and fatty acyl modification. Sonic (Shh) and Indian (Ihh) Hh’s are the...
most widely expressed vascular Hhs during development, and a genetic deficiency in Shh is embryonically lethal in mice.\textsuperscript{16} Signaling occurs through interaction with the Patched receptors (Ptc1 and 2) that subsequently activate the transcription factors Gli1, Gli2, and Gli3. The downstream targets of the Gli gene products include both Ptc and Gli themselves; thus, Ptc and Gli are both components and targets of the Hh signaling pathway.\textsuperscript{16,17} Several recent observations highlight the involvement of Hh in the development of retinal microvasculature.\textsuperscript{5} Activation of Shh signaling in vivo results in the induction of robust angiogenesis in mice,\textsuperscript{18} and in vitro studies have shown that Shh signaling promotes vascular smooth muscle cell (vSMC) growth and survival.\textsuperscript{11,19}

Pathologic changes in the structural integrity of the retinal microvasculature are induced, in part, by changes in the mechanical burden on cells and the subsequent activation of discrete signaling pathways that govern retinal pericyte and endothelial cell growth and survival.\textsuperscript{20–22} Changes to the biomechanical microenvironment can result in substantial changes in retinal pericyte\textsuperscript{22,23} and endothelial\textsuperscript{24,25} proliferation and apoptosis. In this context, we have recently demonstrated that changes in the mechanical microenvironment of retinal endothelial and pericyte populations lead to changes in their proliferative capacity in vitro.\textsuperscript{26}

The purpose of the present study was to evaluate the role of Hh and Notch signaling components in controlling retinal microvascular cell apoptosis in static conditions and after exposure to varying flow environments in vitro.

\section*{MATERIALS AND METHODS}

\subsection*{Materials}

All chemicals were of the highest purity commercially available and were purchased from Sigma Aldrich (Poole, UK) unless otherwise specified.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Figure1.png}
\caption{Immunocytochemical staining of Notch and Hh components in (A) BREC and (B) BRP cells. Notch1 and -3, and Hrt1, -2, and -3, and Ptc1 with corresponding DAPI nuclear staining. Control IgG, von Willebrand Factor (vWF), and α-actin are also shown. Magnification, \( \times 20 \).}
\end{figure}
stated. Antibodies against Sonic, Indian, Ptc1 and Gli2, and Notch components (Hrts, and Notch1 and -3 ICs [intracellular receptor domains]) were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

**Cell Culture**

Bovine retinal microvascular endothelial cells (BRECs) and bovine retinal pericytes (BRPs) were kindly donated by Alan Stitt (Queens University, Belfast). BRECs were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1× insulin-transferrin-sodium selenium (ITS; Sigma-Aldrich, Deisenhofen, Germany). BRPs were grown in DMEM supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1× ITS. Both cell cultures were maintained in a humidified atmosphere of 5% CO2/95% air and routinely used between passages 5 to 9, as previously described.26,27 The purity of cultures was performed at the start of the experiment using specific immunocytochemical analysis for each cell type and the purity confirmed at >99%. There was no difference in the purity after the 72-hour culture period when these cultures were routinely checked.

**Figure 2.** Immunohistochemical staining of Notch and Hh components in human retina. Immunohistochemical DAB staining of the human retina and optic nerve head (ONH) for Notch1, Hrt-2, and Ihh (arrows, DAB) within vessels of the prelaminar region (PLR), retrolaminar region (RLR), and inner vascular plexus (IVP), respectively, with Hrt-3 staining posterior to the optic nerve head (ONH). The ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and ganglion rod and cone layer (GCL) are also shown. **Black arrows:** positive staining; **white arrows:** negative. Magnifications are shown.
Co-culture Studies

Perfused transcapillary co-cultures of BRPs and BRECs were established as previously described.28,27 Briefly, the perfused transcapillary culture apparatus (CellMax QUAD artificial capillary culture system; Spectrum Laboratories Inc., Santa Clara, CA) consisted of an enclosed bundle of 50 semipermeable, coated (Pronectin-F; Sanyo Chemical Industries, Ltd., Kyoto, Japan) polypropylene capillaries (capillary length, 13 cm; outer diameter, 630 μm; wall thickness, 150 μm; luminal area, 70 cm²; outer surface area, 100 cm²; extracapillary volume 1.4 mL; 95% molecular weight cutoff [MWCO], 0.5 μm) through which medium from a reservoir is pumped at a chosen flow rate via silicone rubber tubing. By altering the flow rate with an electronic control unit that is housed outside the humidified incubator, varying pulsatile flow rates and hence pulse heights (pressure) can be achieved in this system. Before the addition of cells, the module was equilibrated for 3 days by circulation of culture medium through the capillaries and tubing. BRPs were harvested by adding 0.125% trypsin-EbDTA and injected into the extracapillary space (ECS) at a density of 2 × 10⁴ cells/cm² by a double-syringe method.28,27 The cells were allowed to adhere for 3 hours, after which the pump was set to low flow (0.3 mL/min; pulse pressure, 6 mm Hg; stress shear, 0.5 dyn/cm²) and returned to the incubator for 3 days. BRECs were introduced into the luminal compartment using the double-syringe method at a density of 2 × 10⁴ cells/cm² and allowed to attach for 3 hours before the medium was circulated at low flow for a further 3 days. Low serum (1%) was used to enhance BREC attachment to the cell culture pronectin-coated capillaries. In addition, to prevent BRECs from being flushed out of the capillaries and to promote their adherence immediately after cell loading, we rerouted the perfusion medium (now also containing 1% FBS) for 6 hours via the ECS, using the side ports. To obtain "high flow," the flow rate was increased steadily over approximately 5 hours until the desired high flow rate was reached (t = 0). After 72 hours exposure to flow, the cells were harvested from their separate compartments by first washing the cells with Hanks’ balanced salt solution (HBSS), by using the double-syringe method and removing the remaining cells by treatment with 0.125% trypsin-EbDTA. Pulse pressures were monitored simultaneously, intraluminally at the inlet port and extraluminally (ECS) at the side port, by using pressure transducers connected to a recorder (models 7 and 7E; Grass-Telefactor Instrument Co. Warwick, RI). In the present study, the cells were exposed to low (0.3 mL/min; 6 mm Hg; 0.2 Hz; 0.5 dynes/cm²) and high (25 mL/min; 56 mm Hg; 2 Hz; 23 dynes/cm²) pulsatile flow. It is important to note that the BRECs seeded within the lumen were exposed to fluid shear stress at the indicated levels. In contrast, the BRPs seeded on the outside of each capillary (ECS) were predominantly exposed to the cyclical transmural pressures generated by the pulsatile flow.

Western Blot Analysis

Proteins from cell lysates (10–30 μg) were resolved on SDS-PAGE (12% resolving, 5% stacking) before transfer onto nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK), as described previously.10 Nonspecific protein binding was blocked by a 90-minute incubation in PBS-T (phosphate-buffered saline, 0.1% Tween-20) containing 5% (wt/vol) nonfat skim milk and 2.5% BSA. Membranes were then incubated at 4°C overnight with one of the antisera described above diluted in PBS-T (1:200–1:500) with 2.5% BSA. Membranes were incubated for 60 minutes at room temperature with horseradish peroxidase–conjugated rabbit polyclonal or mouse monoclonal IgG antibody (1:1000–1:5000; GE Healthcare, Piscataway, NJ). Scanning densitometry was performed with image-analysis software (Image); developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), with values normalized for loading using Ponceau staining for total protein.

**Figure 3.** Inhibition of Hh and Notch signaling promoted BRECs and BRP apoptosis. (A) Recombinant Shh (5 μg/mL) stimulation of Ptc1 mRNA levels in BRECs in the absence or presence of cyclopamine (40 μM) after 24 hours. *P < 0.05 vs. control. #P < 0.05 vs. Shh alone. (B, C) Percent apoptosis in BRECs and BRPs after Hh inhibition with cyclopamine (40 μM) for 24 hours. *P < 0.05 vs. control. (D) The level of Notch1 and -3 ICs in the absence or presence of DAPT (10 μM) for 24 hours. Equal loading and transfer of proteins was confirmed by Ponceau S staining. (E) Ratio of change of apoptosis in BRECs and BRPs after Notch inhibition with DAPT for 24 hours. The number of apoptotic nuclei was determined by FACS analysis and is the mean ± SEM of three independent experiments. *P < 0.05 vs. control.
Immunocytochemistry

The cells from static cultures were fixed and permeabilized in methanol (−20°C, 10 minutes), and subsequently rehydrated in 1× PBS/3% BSA (10 minutes), as previously described.1 The primary antibodies described above were incubated for 3 hours at room temperature (1:100–1:200) and FITC-conjugated secondary antibodies for 1 hour at room temperature.

Immunohistochemistry

Normal human donor eyes were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). The average age of the donors was 69 ± 2.9 years (67% male). Approval was also obtained from the Mater Misericordiae Hospital’s Ethics Committee (Dublin, Ireland) for the procurement of these tissues, and the donor eyes were managed in accordance with the Declaration of Helsinki guidelines for research involving human tissue. Globes were dissected into two, processed and embedded. Briefly, the globes were fixed in 10% formalin within 20 hours of death and placed in 4% paraformaldehyde before the tissues were processed and embedded in paraffin (TP 1020 processor; Leica Microsystems, Wetzlar, Germany). Several 5-μm sections were cut on a microtome (RM 2135; Leica Microsystems), dried on poly-L-lysine-coated glass slides, and baked overnight at 50°C. Endogenous peroxidase activity was quenched by incubation with 0.3% H₂O₂ in 100% methanol for 30 minutes. Nonspecific binding was blocked in 1.5% normal serum (Vectastain Elite kit diluted in PBS; Vector Laboratories, Peterborough, UK) before the sections were incubated with the primary antibodies described above (1:100–1:500), washed, and incubated with the appropriate biotinylated secondary antibody (1:500; Vectastain kit) for 1 hour at room temperature. The slides were washed and exposed to diaminobenzidine (DAB) chromogen for up to 10 minutes. The sections were counterstained with hematoxylin for 30 seconds, washed in water for 5 minutes. The slides were rinsed with methanol and cleared with xylene (three separate washes). The slides were then mounted using DPX mounting medium.

Proliferation and Apoptosis Assay by Fluorescence-Activated Cell Sorting

Cell proliferation and apoptosis was determined by FACS analysis (Vybrant CFDA-SE. Cell Tracer Kit and the Vybrant Apoptosis Alexa Fluor 488 Annexin V kit; Molecular Probes, Eugene, OR).

Figure 4. Recombinant Shh stimulation of HB and Notch target genes in BRECs and BRPs and promotion of BREC and BRP apoptosis. Recombinant Shh (5 μg/mL) stimulation of (A) Ptc1 and Gli2, (B) notch1 and notch3, and (C) Bcl-2, Bcl-xL, and Bax mRNA levels in BRECs and BRPs in the absence or presence of cyclophamide (40 μM) after 24 hours. The data represent the mean ± SEM of three independent experiments. *P < 0.05 vs. control, $ vs. Shh alone.
Fluor 488 annexin V and propidium iodide Assay Kit 2, respectively, on a FACScan flow cytometer (BD Biosciences, Dublin, Ireland). The cells were designated as viable, apoptotic, or necrotic. The cells staining with PI in the flow preparations were predominantly those in late apoptosis, as the cells undergoing primary necrosis disintegrated during detachment and staining.

**Plasmid Expressing Vectors and Plasmid Preparation**

The Epstein Barr virus–encoded gene product RPMS-1, which binds CBF-1/RBP-Jκ, was used to inhibit Notch-dependent CBF-1/RBP-Jκ signaling and was the kind gift of Paul J. Farrell (Ludwig Institute for Cancer Research, Imperial College School of Medicine, London, UK). Plasmids were prepared for transfection according to the manufacturer’s instructions, with a kit (Midi Kit; Qiagen, Valencia, CA), as described previously. The cells were co-transfected with a plasmid-encoding puromycin resistance (pPGKpuro) before puromycin resistance cells were selected and pooled as previously described. The resultant transfection efficiency was greater than 90%.

**Quantitative Real-Time RT-PCR**

Quantitative real-time (qRT-PCR) was performed (Rotor Gene model RG-3000; Corbett Research, Sydney, NSW, Australia) and the SYBR green PCR kit (Qiagen), as described previously. The gene-specific oligonucleotide sequences for Notch and Hh components were as described elsewhere.

**Data Analysis**

Results are expressed as the mean ± SE. Experimental points were performed in triplicate, with a minimum of three independent experiments. Unpaired Student’s t-test or Wílcoxon’s signed-rank test when control values were adjusted to 1, were used for comparison of groups. P < 0.05 was considered significant.

**RESULTS**

**Components of the Notch and Hh Signaling Pathways Are Present in BRECs and BRPs**

Cells derived from bovine retinas were routinely characterized to confirm a pure culture of either pericytes (BRPs) or endothelial cells (BRECs). The BRECs stained positively for von Willebrand factor (Fig. 1A), whereas the BRPs stained for the contractile protein α-smooth muscle cell–specific actin (Fig. 1B; α-actin) and NG2 (data not shown). The pericytes were also identified and distinguished from the BRECs by their size and distinct morphology and by the lack of staining with antisera to von Willebrand factor, endothelial nitric oxide synthase (eNOS) and the astrocyte-specific marker glial fibrillary acidic protein (GFAP; data not shown). The purity

**FIGURE 5.** Pulsatile flow modulated apoptosis in BRECs and BRPs. (A, D) Change in the number of apoptotic nuclei in BRECs and BRPs after exposure to low (0.3 mL/min) and high (25 mL/min) flow after 72 hours. Control cells were exposed to 1% FCS. *P < 0.05 vs. low flow. (B, E) The level and change in Bcl-2 and Bax protein levels and (C, F) the change in Bcl-2, Bcl-xL, and Bax mRNA levels in BRECs and BRPs in the same conditions as in (A) and (D). Data are representative immunoblots and the mean of results in three independent experiments. *P < 0.05 vs. low flow.
of cultures was routinely shown to be >99%. Semiquantitative immunocytochemical analysis of BRECs and BRPs in culture revealed the presence of intracellular receptor domains (IC) of Notch1 and -3 receptors and Notch target gene products (Hrt-1, -2, and -3; Fig. 1) in conjunction with the transmembrane Hh receptor and target gene Patched (Ptc1) (Fig. 1). The intracellular locations for these proteins corresponded with previous reports for vascular cells.10,11,19

Briefly, Notch1 and 3IC were predominantly located in the nuclei of BRECs (Fig. 1A), whereas both the cytoplasm and the plasma membrane of BRPs were stained for both receptors (Fig. 1B). Membrane-tethered Notch1 and 3IC constituted a small proportion of the overall cellular distribution of Notch IC in the BRPs and BRECs. All Notch target gene products exhibited a strong nuclear localization pattern within the BRECs (Fig. 1A), whereas BRPs exhibited a greater degree of cytoplasmic and plasma membrane staining for Hrt-2, and -3 (Fig. 1B). The Hh target gene product Ptc1 was also present on BRECs and BRPs and exhibited a similar vesicular cytoplasmic staining pattern in the BRPs, as reported previously for vSMC.11 Furthermore, the presence of protein and mRNA transcripts in BRECs and BRPs for Notch receptors (Notch1 and Notch3), Notch target gene products (Hrt-1, Hrt-2, and Hrt-3) and the Hh components Sbb and Ibb and Ptc1 and Gli2 mRNA was confirmed by Western blot and qRT-PCR analysis, respectively (data not shown).

Immunohistochemical staining for Notch and Hh signaling components were performed on formalin fixed, paraffin wax-embedded sections of human retina. Three different regions in the optic nerve head (ONH) were identified: (1) the prelaminar region (PLR), localized at the level of the choroid and retina, which includes the superficial nerve fiber layer and is characterized by minimal connective tissue and the absence of clearly myelinated axons; (2) the lamina cribrosa (LC), localized at the level of the sclera and characterized by large connective tissue septa; and (3) the retrolaminar region (RLR), localized outside the eye and characterized by myelinated axons and delicate connective tissue septa. These ONH regions, along with the retina were examined for immunoperoxidase staining. The majority of PLR vessels were positive for Notch1 IC (Fig. 2). Similarly, immunostaining of these vessels for the products of the Notch target genes Hrt-2 and -3 was positive (Fig. 2). Some smaller vessels that are found in the retina and the RLR were also positive for Notch1 IC and its target genes. In contrast, the PLR vessels of the ONH were negative for the Hh morphogens and ligands, Ihh (Fig. 2) and Shh (data not shown). Furthermore, no immunoreactivity was observed for Ihh in RLR vessels. However, a large number of interconnecting vessels between the two layers of vasculature within the retina—the superficial and the deep or inner vascular plexus within the retina stained positively for Ihh (Fig. 2).

**Inhibition of Hh and Notch Signaling Promotes Apoptosis in BRECs and BRPs**

The functional significance of Hh and Notch components in BRECs and BRPs was confirmed after inhibition of each pathway with cyclopamine and DAPT, respectively. The inhibitory effect of cyclopamine, a naturally occurring steroidal alkaloid that inhibits the Hh pathway by interacting with the Hh signaling protein Smoothened (Smo), was confirmed in BRECs (Fig. 3A) and the BRPs (data not shown) since Shh (3 μg/mL) stimulated Ptc1 mRNA levels in the BRECs were attenuated after treatment with cyclopamine (40 μM) for 24 hours (Fig. 3A). In parallel cultures, cyclopamine (40 μM) promoted a significant increase in BRECs and BRPs apoptosis (Fig. 3B). The inhibitory effect of DAPT, a γ-secretase inhibitor that attenuates Notch receptor cleavage and processing, was also confirmed in the BRPs (Fig. 3C) and the BRECs (data not shown), since DAPT (10 μM) decreased Notch1 and -3 IC protein levels in the BRPs after a 24-hour treatment (Fig. 3C). In parallel cultures, DAPT promoted a significant increase in BREC and BRP apoptosis (Fig. 3D). The inhibitory effect of cyclopamine on Hh signaling was further validated in vitro after treatment of cells with tomatidine (40 μM), a steroidal alkaloid structurally similar to cyclopamine that does not inhibit the Hh pathway (data not shown).

**Shh Protects against Serum-Deprivation–Induced Apoptosis and Activates Notch Signaling in BRECs and BRPs**

Ptc1 and Gli2 are both components and transcriptional targets of the Hh signaling pathway. Activation with recombinant Shh protein (3 μg/mL) resulted in a significant increase in Ptc1 and Gli2 target gene mRNA levels in both BRECs and BRPs. Treatment of both BRECs and BRPs with Shh in the presence of the Shh inhibitor cyclopamine (40 μM) resulted in a significant decrease in Shh induction of Ptc1 and Gli2 mRNA levels in each cell type (Fig. 4A). Shh stimulation of Ptc1 mRNA was more robust in the BRECs when compared with that in the BRPs, whereas Shh stimulation of Gli2 mRNA levels appeared to predominate in the BRPs. To investigate whether stimulation of Hh signaling resulted in increased expression of Notch components, real-time PCR analysis of BREC and BRP mRNA

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**Figure 6.** Pulsatile flow modulated Notch and Hh component mRNA levels in BRECs and BRPs. The change in (A) Ptc1, Gli2, Smo, Sbb, Ihh, notch1, and notch3 mRNA levels in BRECs and (B) BRPs after exposure to low (0.3 mL/min) and high (25 mL/min) flow after 72 hours. Data are the mean of the results in three independent experiments. *P < 0.05 vs. low flow.
samples was performed after activation of the cells with recombinant Shh. As Notch receptors themselves are also downstream targets for CBF-1/RBP-Jk–dependent Notch signaling, recombinant Shh increased the expression of Notch1 and -3 receptor mRNA levels in the BRECs and Notch1, but not Notch3 in the BRPs (Fig. 4B). Attenuation of this response by the addition of cyclopamine (40 μM) confirmed that Shh acts to stimulate Notch by derepressing the Ptc1-Smo complex (Fig. 4B). In parallel cultures, recombinant Shh promoted antiapoptosis gene expression (Bcl-2 and Bcl-xL), whereas simultaneously inhibiting proapoptotic marker expression (Bax) in both BRECs and BRPs, an effect that was inhibited by Shh inhibition with cyclopamine (Fig. 4C; 40 μM).

**Pulsatile Flow Modulates Apoptosis and the Expression of Hh and Notch Components in BRECs and BRPs**

BRP/BREC co-cultures were exposed to low or high pulsatile flow for 3 days, as described previously. Pulsatile flow decreased the number of apoptotic nuclei in BRECs concomitant with a significant increase in Bcl-2 protein and mRNA levels, while reducing Bax protein and mRNA levels (Figs. 5A–C). In contrast, pulsatile flow increased BRPs apoptosis concomitant with a significant decrease in Bcl-2 protein and mRNA levels, while enhancing Bax protein and mRNA levels (Figs. 5D–F).

The effect of pulsatile flow on components of the Notch and Hh signaling pathways was also determined by Western blot and quantitative real-time PCR analysis. When compared with low flow, high pulsatile flow increased mRNA levels of Notch and Hh pathway components in BRECs (Fig. 6A) while decreasing mRNA levels of the majority of Notch and Hh genes analyzed in the BRPs (Fig. 6B). Parallel immunoblot studies confirmed a preferential increase in Notch and Hh signaling proteins in the BRECs and a decrease in these proteins in the BRPs in response to flow (data not shown). To determine whether the changes in BRP Notch1 levels were dependent on signaling between BRECs and BRPs, cells were exposed to high flow in the absence of BRECs for 3 days. Notch1 IC levels decreased 0.61 ± 0.07-fold (n = 3) relative to low flow, similar to the Notch decrease when BRPs were examined in co-culture.

**Hh Inhibition Reverses Pulsatile Flow–Induced Changes in BREC Apoptosis**

To elucidate the direct effect of Hh inhibition on BRECs exposed to pulsatile flow, experiments were performed on monocoltured BRECs. Pulsatile flow increased Hh target gene mRNA levels (Gli2 and Ptc1; Figs. 7A, 7B) concomitant with a significant decrease in BREC apoptotic nuclei (Fig. 7C) and an increase in Bcl-2 mRNA levels (Fig. 7D) in monocultured BRECs, in common with what was observed in co-cultures. Moreover, the anti-apoptotic effects of flow on the number of apoptotic nuclei in BRECs and the increases in Bcl-2 mRNA levels were attenuated by inhibition of Hh signaling with cyclopamine (Figs. 7C, 7D), concomitant with a significant reduction in Hh target gene Ptc1 and Gli2 expression (Figs. 7A, 7B).

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**FIGURE 7.** Hh inhibition reversed pulsatile flow–induced changes in BREC apoptosis. The change in (A) Ptc1 and (B) Gli2 mRNA levels in monocoltured BRECs after exposure to low (0.3 mL/min) and high (25 mL/min) flow after 72 hours in the absence or presence of cyclopamine (40 μM). (C) The change in the number of apoptotic nuclei in BRECs and the change in Bcl-2 mRNA levels in monocoltured BRECs in the same conditions. Data are the mean of the results in three independent experiments. *P < 0.05 vs. low flow, $P < 0.05$ vs. (−) cyclopamine.
Notch Inhibition Reverses Pulsatile Flow–Induced Changes in BREC Apoptosis

In parallel studies, inhibition of Notch signaling after ectopic expression of the CF-1/RBP-Jk binding protein RPMS-1 reversed the flow-induced decrease in the number of BREC apoptotic nuclei (Fig. 9A) concomitant with a reversal of the flow-induced decrease in Bax mRNA levels (Fig. 9B) when compared to mock controls. The functional activity of RPMS-1 on CF-1/RBP-Jk dependent promoter transactivation was confirmed (data not shown). Furthermore, RPMS-1 expression preferentially inhibited Bcl-xL, and Notch1 mRNA levels but not Bcl-2 or Somo mRNA levels (Fig. 9C).

DISCUSSION

Ocular neovascularization associated with proliferative diabetic retinopathy (DR) and age-related macular degeneration (AMD) is the leading cause of severe visual loss in adults in developed countries.29 Notch and Hh signaling molecules have been implicated in a multiplicity of developmental and postnatal processes that govern vasculogenesis and remodeling.3-5 Using a repertoire of antibodies targeting Notch receptor and ligand domains, Notch components have been shown to reside in tip cells, capillaries, and arterial vessels during retinal vascular development.30 Hh signaling is also present and is considered a prerequisite for normal retinal neuronal development.31-33 Its role in physiological and pathologic ocular neovascularization has recently been highlighted where Hh signaling components were upregulated in the retina of animal models with neovascular disease while cyclopamine inhibited the development of the retinal microvasculature.5 In this context, we have demonstrated that Notch and Hh components are functionally active in adult retinal microvascular endothelial and pericyte cells in vitro. Moreover, pulsatile flow promotes endothelial cell survival while enhancing pericyte apoptosis through modulation of Notch and Hh pathways. These putative interactions may have important implications for the pathogenesis of retinopathies.

The presence of Notch and Hh signaling components was confirmed within human retinal microvasculature in vivo and in isolated BREC and BPC cultures. Inhibition of Notch signaling with DAPT, a γ-secretase inhibitor, resulted in a marked increase in endothelial and pericyte apoptosis in serum-stimulated cells, confirming a functional role for Notch in dictating retinal microvascular fate in vitro. Similarly, cyclopamine inhibition of Hh signaling confirmed a similar prosurvival role for Hh signaling in serum-deprived cells under static conditions. Moreover, Hh stimulation of retinal endothelial and pericyte cell survival was associated with an increase in Notch target gene expression since Notch1 mRNA levels (a Notch target gene28) were attenuated after Hh inhibition in these cells. It is important to note that the molecular targets for cyclopamine and RPMS-1 are different, in as much as cyclopamine blocks CBF-1/RBP-Jk-dependent Notch signaling downstream whereas RPMS-1 blocks all Notch signaling that is CBF-1/RBP-Jk dependent. Indeed, we have also examined Hh inhibition of flow-induced changes in Notch components and demonstrate that unlike Notch1 receptor mRNA levels, cyclopamine at the same concentration failed to inhibit the flow-induced increase in BREC Notch3 mRNA levels. In contrast, RPMS-1 blocked both Notch1 and -3 mRNA levels in these cells (data not shown).

The similar level of apoptosis in response to RPMS-1 under low and high flow conditions is not wholly unexpected. RPMS-1, as an inhibitor of CBF-1/RBP-Jk-dependent Notch target gene expression, mimics the actions of R218H (a mutant CBF-1/RBP-Jk) in these cells (data not shown). In this context, even if Notch1 and -3 IC receptor expression is differentially regulated...
under low- and high-flow conditions, these differences become less significant to the final apoptotic response when downstream CBF-1/RBP-JΔ dependent target gene expression is blocked with RPMS-1.

Previous studies have suggested a significant role for Shh stimulation of Notch target genes (Hes-1) that require the Shh effector, Gli2 in retinal explants derived from postnatal mice.34 A role for Hh signaling in the adult retina is further indicated by sustained Shh expression in both retinal ganglion cells (RGCs) and a subset of inner nuclear layer amacrine cells, and of Ptc1 expression in Müller cells35 with preliminary data supporting Shh and Ptc1 expression by RT–PCR analysis in adult human retina.35 Our data support an important functional role for Hh signaling in controlling microvascular retinal cell apoptosis through Notch signaling since the majority of PLR vessels at the ONH were positive for Notch1 and Notch target genes (Hrt-1, Hrt-2, and Hrt-3), whereas interconnecting vessels stained positively for Ihh. The distinct pattern of Ihh in the smaller retinal capillaries, but not in the larger veins or arteries is noteworthy. This apparent pattern may be due to pericyte or pericyte-EC cross-talk in the microvascular compartment. In situ hybridization studies are needed, to further determine the source of the Notch and Hh ligands within the retinal microvasculature—in particular for the secreted Hh ligands, as positive staining may represent ligand bound to receptors on receiving cells or bound in the extracellular matrix. Furthermore, localization studies using fluorescent analysis co-staining with EC, mural or astrocytic markers will reveal more precise information on the location of Hh and Notch ligands and receiving cells. These studies are currently under investigation.

Vascular cell growth is a critical feature of retinal microvascular dysfunction in various retinopathies in which endothelial cell proliferation and pericyte loss prevail.36–38 Perturbations in the retinal microcirculation may be a causative factor for vascular dysfunction since retinas from DR patients are highly sensitive to changes in blood glucose levels, systemic hypertension and exhibit impaired flicker-induced vasodilation.39 During the latter stages of DR, loss of vessel homeostasis leads to regional vascular changes, with both vessel regression and neovascular growth of immature vessels lacking pericyte coverage, each of which implies an alteration in endothelial and pericyte cell apoptosis and proliferation.40 A significant corre-

**Figure 9.** Notch inhibition reversed pulsatile flow–induced changes in BREC apoptosis. (A) Change in the number of apoptotic nuclei, (B) in Bax mRNA levels, and (C) in Bcl-2, Notch1, Bcl-2 and Smo mRNA levels in monocultured BREC after exposure to low (0.3 mL/min) and high (25 mL/min) flow after 72 hours, with or without ectopic expression of the CBF-1/RBP-JΔ inhibitor RPMS-1. Data are the mean of the results in three independent experiments. *P < 0.05 vs. low flow, #P < 0.05 vs. (–) RPMS-1 empty vector mock control.
lation is apparent between the progression of diabetic background retinopathy and increased retinal blood flow.\textsuperscript{41,42} In this context, we have recently demonstrated that increased pulsatile flow modulated retinal microvascular endothelial and pericyte cell growth in vitro by altering the release of endothelial derived vasoreactive substances that control proliferation and apoptosis.\textsuperscript{26}

In the present study, we demonstrated an additional functional role for Hh and Notch signaling components in controlling retinal microvascular cell apoptosis under static conditions and after exposure to increased flow environments in vitro. Although pulsatile flow was ineffective at altering endothelial proliferation rates,\textsuperscript{25} the level of apoptosis decreased concomitant with enhanced Hh and Notch signaling. Furthermore, the Hh-dependent promotion of endothelial cell survival was Notch dependent, since treatment with cyclopamine or ectopic expression of a CBF-1/RBP-Jk inhibitor (RPMS-1) reversed the flow-induced survival response while concomitantly inhibiting Notch1 mRNA levels. Moreover, the preferential inhibitory effects of RPMS-1 on Bcl-x\textsubscript{L} and Notch1 mRNA levels but not on Bcl-2 or Smo further suggests that expression of RPMS-1 and Notch inhibition is preferentially targeting specific genes under flow and not gene transcription in general. Recent studies have also shown a similar protective effect of ganglion-derived Shh in chronic hypertensive retinas where Shh promoted the survival of damaged RGCs in chronic hypertension.\textsuperscript{44} Similarly, in the absence of Shh, p53-induced apoptosis and inhibition of retinal cell proliferation, cell-cycle exit, and differentiation in zebrafish occurred unabated.\textsuperscript{45} Our findings suggest that the Shh signaling pathway may also exert a similar vascular protective effect for retinal microvascular endothelial cells in various retinopathies aggravated by hypertension.

Diabetic retinopathy is morphologically characterized by pathologic changes in retinal capillaries and is associated with a characteristic loss of pericytes and the progressive occlusion of capillaries.\textsuperscript{46} The underlying mechanism(s) responsible for pericyte loss in DR are complex and incompletely understood. Retinal pericyte loss due to decreased proliferation\textsuperscript{46} and increased apoptosis is associated with higher flow rates apparent during early DR and is accompanied by significant decrease in Hh and Notch signaling components in these cells in vitro. Activation of the Hh signaling is an important feature of distinctive pericytic phenotypes.\textsuperscript{47} For example, Shh produced by the hindbrain choroid plexus epithelium induces the extensive vascular outgrowths in mice during development, with pericytes transducing the Hh signaling.\textsuperscript{47} Pericytes are the capillary counterparts to VSMCs, and it is noteworthy that Hh signaling is also significantly regulated within macrovascular beds promoting VSMC proliferation and survival.\textsuperscript{11,19} Moreover, Hh expression is diminished by biomechanical stimulation in vitro and in vivo and may play a fundamental role in macrovascular arterial remodeling.\textsuperscript{11,19} The fact that flow-induced apoptosis of retinal pericytes was associated with a decrease in Hh signaling is consistent with a reduction in Notch signaling further highlights the importance of these pathways in dictating pericyte apoptosis. In addition, recent data also suggest that altered migration may also contribute to pericyte loss in DR\textsuperscript{45} and that this mechanism is regulated by signaling via the Ang-2/Tie-2 pathway, known downstream effectors of Hh and Notch signaling.\textsuperscript{48} Although early DR is associated with enhanced retinal blood flow, more recent studies report significantly decreased flow velocities in the retinal arterioles and venules of patients with diabetes compared with healthy control subjects, supporting the view of abnormal vessel function in retina with nonproliferative diabetic retinopathy.\textsuperscript{49} Our data also confirmed that flow is protective for endothelial cells since high flow inhibited BREC apoptosis. This finding may have important repercussions for DR and AMD, where reduced retinal blood flow is associated with the development and progression of the disease where any decrease in flow is likely to be a proapoptotic stimulus for BREC.

In conclusion, we have shown for the first time that Hh and Notch components are present within the PLR vessels at the ONH of human retina. Our studies demonstrate Hh and Notch signaling pathways in microvascular endothelial and pericyte cells co-operate to control cell survival in response to flow. Alterations in the microenvironments of these cells due to changes in retinal blood flow may induce significant changes in Hh and Notch signaling in vivo potentially having major repercussions of retinal microvascular cell survival. Elucidation of the functional role of these interactions in the pathogenesis of retinopathies in patients may prove invaluable in the treatment of these conditions. Future studies are needed to determine whether individual Hh and Notch components are differentially regulated within the retinal microvasculature in various proliferative retinopathies and underscore the important implications for vessel growth and regression during retinal vascular pathogenesis.

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